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A Method for the Evaluation of the Efficiency of Signal Sequences for Secretion and Correct N-Terminal Processing of Human Parathyroid Hormone Produced in *Escherichia coli*

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Expression plasmids have been constructed for evaluation of different signal sequences for secretion and correct amino terminal processing of foreign proteins expressed in *Escherichia coli*. cDNA representing the N-terminal region (1-37) of human parathyroid hormone was inserted between DNA coding for two different forms of the signal sequence and two IgG binding domains (ZZ) derived from *Staphylococcal* protein A. The expression products were secreted to the periplasm and even to the growth medium and were easily purified by affinity chromatography using the ZZ part as a specific handle. Further analyses showed that the expression products were correctly processed to the mature protein hPTH(1-37)ZZ in a construct where the wild type signal sequence of *Staphylococcus* protein A was used. When a mutated signal sequence which lacks the normal cleavage site was employed, the fusion protein was not cleaved. Since signal sequences seem to be processed in the correct way in this system, we conclude that the general design of this type of expression vector is well suited for studying the N-terminal processing and secretion of heterologous proteins in *E. coli*. © 1992 Academic Press, Inc.

Most secreted proteins from both eukaryotes and prokaryotes contain an amino terminal extension (1-3). This so called signal sequence serves to lead the secreted proteins into the secretory pathway, and during the secretion process it is removed by a specific signal peptidase (4). Bacterial signal peptidases are often able to cleave off the signal sequence of exported proteins from other prokaryotic species (5). In some cases they also correctly process eukaryotic preproteins (6), as well

as hybrid proteins containing prokaryotic signal peptides fused to eukaryotic proteins or vice versa (7,8).

Escherichia coli possesses two cell membranes, and all the proteins in the outer membrane and periplasmic space belong to the category of secretory proteins (9). *E. coli* contains thousands of different intracellular proteins and approximately 50 different proteins in the periplasm (10). In contrast, less than 10 proteins are normally translocated to the culture medium, and the mechanism of outer membrane passage remains unclear.

E. coli has been used to achieve high level expression of many heterologous proteins (11,12). In most cases, the products remain in the cytoplasm and must be purified and frequently *in vitro* processed (13,14). In addition to the need for a complex purification scheme there are also other disadvantages of the intracellular localization of expression products, such as the generation of insoluble protein aggregates (inclusion bodies). Moreover, since protein synthesis in prokaryotes is always initiated with *N*-formylmethionine, overproduced proteins often have an extra undesired *N*-terminal *N*-formylmethionine.

Human parathyroid hormone (hPTH)¹ is an 84 amino acids single chain polypeptide secreted from the parathyroid gland and is of central importance in the regulation of calcium homeostasis (15). The amino acid and cDNA sequences of hPTH have been established

¹ Abbreviations used: hPTH, human parathyroid hormone; ZZ, tandem IgG binding domains derived from *Staphylococcus aureus* protein A; HPLC, high-pressure liquid chromatography; HSA, bovine serum albumin; OD, optical density; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

(16-19), and it is well known that most additions or deletions in the N-terminal region substantially reduce the biological activity (15). A recombinant expression system for hPTH should therefore preferably give a product with the correct N-terminal.

We report the construction of expression plasmids where cDNA coding for a part of hPTH is placed between DNA coding for two variants of the signal sequence of *Staphylococcus aureus* protein A and DNA coding for two synthetic IgG binding domains (designated ZZ) (10,20). Both these constructs are under the transcriptional control of the protein A promoter and terminator sequences. *E. coli* cells transformed with these plasmids express truncated hPTH fused to the two IgG binding domains of protein A. The expression products can be quickly purified by IgG affinity chromatography and analyzed for the N-terminal amino acid sequence. This model enables us to study processing and secretion of hybrid proteins expressed in *E. coli*.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other DNA-metabolizing enzymes were from New England Biolabs. ¹²⁵I-labeled anti-rabbit IgG both whole and F(ab)₂ fragments (from donkey) were from Amersham Corp., NH₂-terminal-specific anti-PTH antibody was bought from CHEMICON and ZZ-specific antibody was obtained from Kabi Pharmacia AB. Synthetic hPTH(1-84) was from Sigma and Immobilon polyvinylidene difluoride transfer membrane was from Millipore. The high-pressure liquid chromatography (HPLC) column used was 25 cm × 4.6 mm Vydac reversed phase C₁₈ protein/peptide column.

Bacterial Strains, Plasmids, and Recombinant DNA

Methods

E. coli strain BJ5183 (obtained from Dr. F. Lacroute, Centre de Genetique Molculaire du C.N.R.S., Gif-sur Yvette, France) was used as bacterial host. The protease mutant strains UT5600 and B121 were gifts from Dr. J. Grodberg. DNA sequencing was performed on plasmid DNA with Sequenase (United States Biochemical Corp.) in accordance with the suppliers manual. If not otherwise stated, recombinant DNA methods were performed according to Maniatis *et al.* (21).

The cloning of hPTH has been described elsewhere (19). The construction of the plasmids pSPTH and pKP43PTH where hPTH(1-84) succeeds the signal sequence of protein A and is under the transcriptional control of protein A promoter has been described previously (22). pKP43PTH contains a mutated version of the protein A signal sequence where the normal cleavage site has been deleted and a Pro residue is situated in

the -2 position (Fig. 1A). In comparison, pSPTH contains the entire wild type signal sequence after insertion of a 40-bp oligonucleotide (Fig. 1B) between the *Ava*I/*Nsi*I sites of pKP43PTH.

The final constructs pSPTH37ZZ and pSPTH37ZZ were made by removing the 141-bp *Sty*I/*Xba*I fragment from the plasmids pSPTH and pKP43PTH, respectively, in order to remove the hPTH stop codon and to align the ZZ-part in correct reading frame with the hPTH cDNA (Fig. 1B). *E. coli* clones transformed with pSPTH37ZZ and pSPTH37ZZ were selected by immunological screening for the production of protein A.

Screening for the Production of Protein A

This was performed according to a modified version of the procedure described by Helfman and Hughes (23). Transformed clones (in replicas) were grown to a suitable colony size on nitrocellulose membranes mounted on agar plates containing ampicillin. Bacterial lysis was induced by suspending the replica membranes in a chloroform vapor glass chamber for 60 min. The membranes were washed overnight in a petri dish containing 20 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 µg/ml DNase I, 40 µg/ml lysozyme, and 3% (w/v) HSA and were rewashed the next day in Tris-buffered saline (50 mM Tris-HCl at pH 7.5, 150 mM NaCl) to remove the bacterial debris. The membranes were then incubated with ¹²⁵I-labeled anti-rabbit IgG (0.3 µCi/ml) in Tris-buffered saline containing 3% (w/v) BSA, washed five times in Tris-buffered saline containing 1% Tween 20, and then subjected to autoradiography.

Cell Growth and Preparation of Cellular Fractions

Transformed bacteria were grown at 37°C under vigorous shaking in 2-liter culture flasks containing 500 ml LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, and 10 g NaCl per liter medium) containing ampicillin (0.5 g/liter) and 0.4% (w/v) glucose. Cells were harvested at OD₆₀₀ = 1.6 (exponential phase) or at OD₆₀₀ = 2.5 (stationary growth phase) by centrifugation at 5000g for 20 min, and the supernatant was collected as the growth medium fraction. The periplasmic fraction was prepared by an osmotic shock method (24).

Affinity Purification

Proteins from the growth medium and the different cell extracts were purified by IgG affinity chromatography using fast flow IgG-Sepharose (Pharmacia). The column (0.9 × 3.2 cm) was equilibrated with TST (50 mM Tris buffer, pH 7.4, 0.15 M NaCl, 0.05% Tween) before loading the sample. Fractions prepared from 1 liter of culture were loaded. After loading the column was washed with 200 ml of TST buffer, equilibrated

with 30 ml of 10 mM ammonium acetate, and eluted with 10 ml of 0.2 M acetic acid, pH 3.2 (titrated with ammonium acetate). The flow rate was 3 ml/min, and the elution was followed by monitoring the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed with 15% gels as described by Laemmli (25). An elute volume of 10 ml after IgG affinity chromatography was lyophilized and solubilized in a buffer containing 0.1 M Tris-HCl, pH 7.5, 17% glycerol, 4% SDS, 0.05% bromophenol blue, and 2% β -mercaptoethanol. The samples were incubated in a boiling water bath for 5 min before being loaded on the gel. Electrophoresis was run for 2–3 h at 600 V.

Electroblotting, Immunoprobings, and Amino Acid Sequence Analysis

Proteins separated by SDS-PAGE were electroblotted to 0.45 μ m Immobilon membranes and the filters were stained by Coomassie brilliant blue as described by Høgset *et al.* (22). For immunoprobings, unspecific binding of antibodies was blocked by incubating the membranes overnight with a blocking agent in PBS [10% fetal calf serum for detection of ZZ; 3.5% human IgG for specific detection of the hPTH part (26)]. Antibody incubation and washes were performed as described (27). For visualization of hPTH the primary antibody was N-terminal specific anti-hPTH from rabbit (dilution 1:1000), and the secondary antibody was 125 I-labeled anti-rabbit IgG F(ab)₂ fragments from donkey. To detect the presence of ZZ, antiserum against ZZ was used and detected with whole 125 I-labeled anti-rabbit IgG from donkey. Autoradiography was performed overnight at -70°C with Kodak X-Omat AR5 film and an intensifying screen (Du Pont).

Aminoterminal sequence analysis was performed on blotted and stained proteins as described earlier (19).

Purification of Fusion Proteins by HPLC

The elute from IgG affinity chromatography was further purified by reversed phase HPLC as described by Høgset *et al.* (19). The eluted material was monitored by measuring the absorbance at 220 nm.

RESULTS

Expression Plasmid Construction

Two expression plasmids were constructed as described under Materials and Methods and outlined in Fig. 1B. In the final constructs the ZZ nucleotide sequence is fused in correct reading frame to the 3' end of

the hPTH cDNA. In pSPTH37ZZ the hPTH part is preceded by a nucleotide sequence coding for the normal signal sequence of protein A, while pSPTH37ZZ codes for a mutated signal sequence designed not to be cleaved by signal peptidase. In this construct the normal signal-peptidase-cleavage-site has been removed and a Pro residue has been placed in the -2 position to prohibit normal processing (1,4). Cells transformed by pSPTH37ZZ thus serves as a control to ensure that the fusion proteins are processed in a normal way.

Expression and Purification of ZZ-Containing Gene Products

E. coli strain BJ5183 was transformed with expression plasmids pSPTH37ZZ or pSPTH37ZZ, and the resulting clones were screened for ZZ production as described (Fig. 2). The positive colonies analyzed were all shown to contain the correct plasmids as determined by restriction enzyme and DNA sequence analyses. Clones containing pSPTH37ZZ and pSPTH37ZZ were grown, and the different cellular fractions were prepared as described under Materials and Methods. ZZ-containing polypeptides were affinity-purified on IgG-Sepharose and the eluted polypeptides were analyzed by SDS-PAGE and immunoblotting. For pSPTH37ZZ-containing cells harvested in the exponential growth phase, most of the affinity-purified material was found in the periplasmic fraction (Figs. 3A and 4A). In the growth medium fraction, two bands with estimated molecular weights of 14 and 15 kDa were detected (Fig. 3A, lane 3 and Fig. 4A, lane 4), while in the periplasmic fraction two additional protein bands were observed, at 19 and 15.5 kDa (Fig. 3A, lane 4). The 19-kDa band corresponds to the theoretical size of the intact fusion protein hPTH(1–37)ZZ. No protein bands appeared in a control represented by medium from cells transformed with the plasmid pSPTH (22) expressing hPTH(1–84) without the ZZ domain (Fig. 3A, lane 2). We therefore conclude that IgG-Sepharose affinity chromatography did, as expected, give a very specific purification of ZZ-containing proteins.

Cells containing pSPTH37ZZ that were harvested in the exponential growth phase exhibited four major bands in the medium fraction, migrating at 14, 22, 24, and 26 kDa, respectively (Fig. 3C, lane 1). In the periplasmic fraction only two faint bands were observed, migrating at 14 and 22 kDa (Fig. 3C, lane 2). The 22-kDa band corresponds to the theoretical size of nonprocessed fusion protein [SSPTH(1–37)ZZ], and the presence of this band indicates that the mutated signal sequence is not cleaved off during secretion.

pSPTH37ZZ-containing cells harvested in the early stationary phase, most of the ZZ-containing proteins seemed to be present in the growth medium fraction, but the presumably intact 19-kDa hPTH(1–37)ZZ spe-

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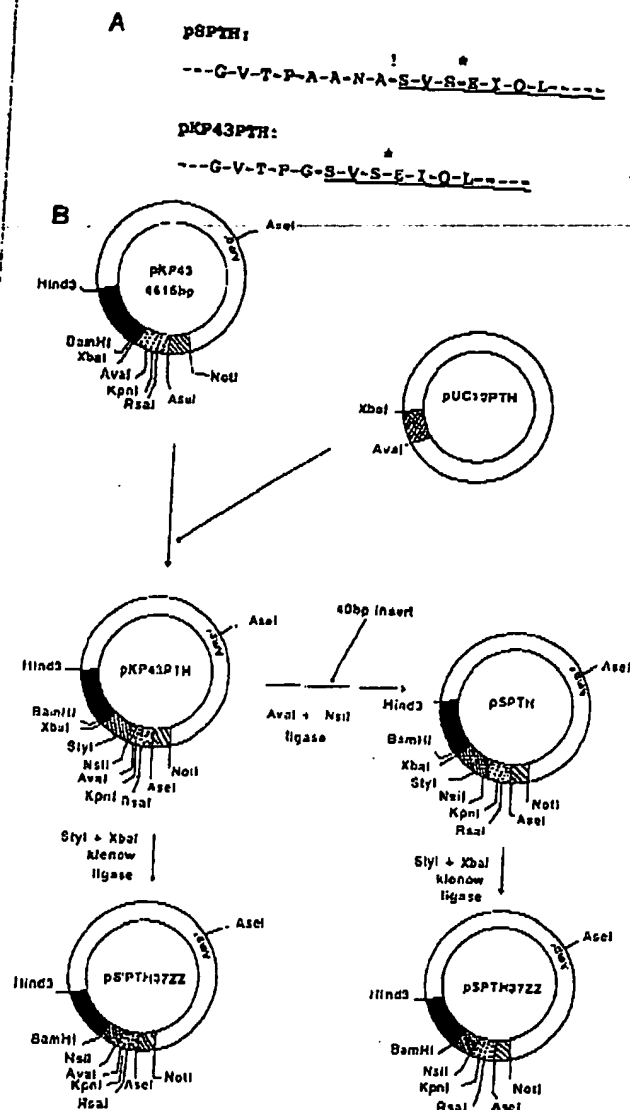


FIG. 1. Comparison of signal sequences encoded by pSPTH and pKP43PTH (A), and schematic drawing of the construction of plasmids pSPTH37ZZ and pSPTH37ZZ (B). (A) Structure of the carboxy-terminal part of the signal sequence encoded by the expression plasmids pSPTH and pKP43PTH. pSPTH contains the natural protein A sequence and in pKP43PTH the normal signal peptidase cleavage site has been deleted (for details see text). Potential signal peptidase cleavage sites according to von Heijne (1984) are indicated with (!) for suggestively preferred sites and (*) for suboptimal sites. The sequence of hPTH is underlined. (B) Plasmid pKP43PTH is made by inserting an Aval/XbaI fragment from pUC19PTH (hatched area) between the Aval and XbaI sites in pKP43 (dark area represents the two IgG binding domains of *Staphylococcal* protein A (ZZ), dotted area represents the signal sequence, and the diagonal area represents the promoter). Removal of the StyI/XbaI fragment from pKP43PTH gave pSPTH37ZZ in which the stop codon of hPTH was removed and the coding region of hPTH was aligned in correct reading frame with the ZZ part. pSPTH, which contained the wild type signal sequence of protein A, was constructed by inserting a 40-bp oligonucleotide between the Aval and XbaI sites in pKP43PTH. Removal of the StyI/

cies could not be detected (Fig. 3B, lane 1). However, two minor bands of 22 and 24 kDa were seen, suggesting the presence of unprocessed fusion protein. Thus at this stage of growth most of the expression products appeared as degraded forms mainly of 15 and 14 kDa (Fig. 3B, lane 1). In the periplasmic fraction of the same culture, only the degraded forms were observed (Fig. 3B, lane 2). Since the greatest amounts of the presumably correctly processed 19-kDa protein were present in exponentially growing cultures, we chose to perform the further experiments on material from such cultures.

Immunoprobings of affinity-purified material from exponentially growing pSPTH37ZZ-containing cells revealed a major hPTH immunoreactive band at 19 kDa in the periplasmic fraction (Fig. 4B, lane 3 and Fig. 5, lane 4 and Fig. 5, lane 1). This strongly indicates that the protein in this band contained the hPTH N-terminal amino acids. In addition there were minor immunoreactive species migrating at 24, 22, and 12 kDa (Fig. 4B, lanes 3 and 4). In *E. coli* transformed with pSPTH37ZZ the main band observed was at 22 kDa (Fig. 5, lanes 3 and 4). This band probably represents an uncleaved fusion protein, indicating that the mutated signal sequence is not cleaved by signal peptidase.

The specificity of the antibody antigen reaction is indicated by the fact that the hPTH standard gave a signal as a positive control (Fig. 4B, lane 1, Fig. 4A, lane 1), while a ZZ standard did not bind to the anti-hPTH antibody (Fig. 4B, lane 2) although it was clearly visible on Coomassie brilliant blue staining (Fig. 4A, lane 2). Several ZZ-containing fragments were also not stained with the anti-hPTH antibody (compare Fig. 4A lanes 3 and 4 with Fig. 4B lanes 3 and 4).

On a parallel filter we also performed immunoprobings for ZZ. Immunoreactive bands migrating at 10, 12, 15, 16.5, and 19 kDa were visible in the periplasmic fractions (Fig. 4C, lane 1), corresponding to the proteins observed by Coomassie brilliant blue staining (Fig. 4A) and confirming the specificity of the affinity purification step. The intracellular fraction of clones containing plasmid pSPTH37ZZ showed very weak bands of ZZ-containing fusion proteins migrating at 19, 18.7, and 15 kDa (data not shown).

Purification of Gene Products by Reversed Phase HPLC

Periplasmic fraction IgG-Sepharose elutes from cultures of pSPTH37ZZ-containing cells were further purified by reversed phase HPLC as described. This revealed three major peaks denoted fractions 20, 22, and 24 as shown in Fig. 6A. These fractions were subjected

XbaI fragment from pSPTH gave rise to pSPTH37ZZ where the hPTH stop codon was removed as described above for pSPTH37ZZ.

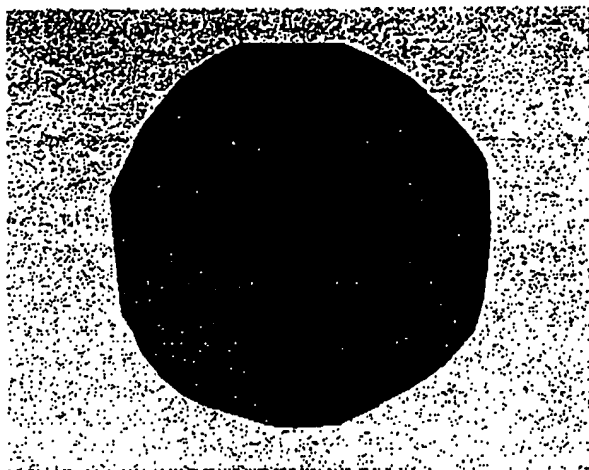


FIG. 2. Autoradiography of protein A producing bacterial colonies. *E. coli* was transformed with pSPT1137ZZ, clones were hand picked, streaked onto a nitrocellulose membrane, and screened for ZZ production by probing with 125 I-labeled anti-rabbit IgG. Negative control clones were streaked on the upper and lower edges (indicated by arrows). The five positive clones analyzed further (see text) are numbered (1-5).

to SDS-PAGE and electroblotting (Fig. 6B). Coomassie brilliant blue staining of the filters showed a protein migrating at 19 kDa in fraction 24 (Fig. 6B, lane 1) and a band at 15.5 kDa in fraction 22 (Fig. 6B, lane 2), whereas proteins migrating at 14 and 15 kDa were observed in

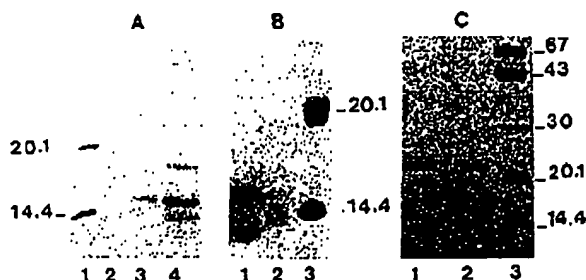


FIG. 3. SDS PAGE analysis of affinity-purified material from different fractions. Comparison of proteins secreted into the growth medium and the periplasmic space during the exponential growth phase of cells transformed with pSPTH37ZZ (A) and pSPT1137ZZ (C) and the stationary growth phase of cells transformed with pSPTH37ZZ (B). Elutes from IgG affinity chromatography were lyophilized, dissolved in 100 μ l distilled deionized water and 5 μ l (for samples from pSPTH37ZZ) or 25 μ l (for samples from pSPT1137ZZ) of the dissolved pellets were subjected to SDS-PAGE, electroblotted, and stained by Coomassie brilliant blue. (A) Lane 1, molecular weight standards; lane 2, control (see text for details); lane 3, growth medium fraction; lane 4, periplasmic fraction. (B) Lane 1, growth medium fraction; lane 2, periplasmic fraction; lane 3, molecular weight standards. The amounts loaded represent 25 ml of culture per lane. (C) Lane 1, growth medium fraction; lane 2, periplasmic fraction; lane 3, molecular weight standards.

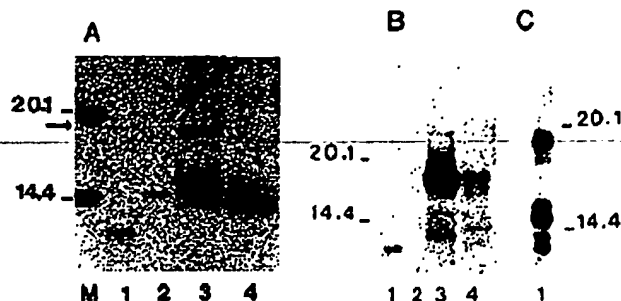


FIG. 4. SDS-PAGE and immunoblotting of affinity-purified material from pSPTH37ZZ-containing cells harvested during exponential growth. Comparison of proteins secreted into the growth medium and periplasmic space. The proteins were visualized by Coomassie brilliant blue staining of filters (A), immunoblotting for hPTH(1-84) standard (1 μ g); lane 2, ZZ standard (1 μ g); lane 3, material from periplasmic fraction; lane 4, material from growth medium fraction. The amounts loaded in lanes 3 and 4 represent 25 ml of culture. (B) Filter from A was immunoprobed with the anti-hPTH antibody. (C) Lane 1, material from the periplasmic fraction was immunoprobed for the detection of ZZ. Positions of molecular weight standards are indicated.

fraction 20 (Fig. 6B, lane 3). Unpurified elutes contained all the bands seen in the different HPLC fractions 20, 22, and 24 (Fig. 6B, lane 5).

N-Terminal Amino Acid Sequence Analysis of Expression Products from Cells Containing pSPTH37ZZ

To further analyze the main expression products, the 19-, 15-, and 14-kDa bands from the HPLC-purified material were cut out from the filter and subjected to N-

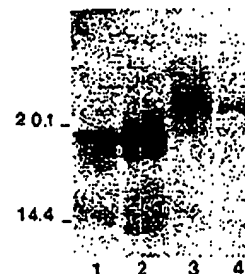


FIG. 5. Comparison of proteins in the growth medium and periplasmic fractions expressed from the plasmids pSPTH37ZZ and pSPT1137ZZ. Affinity-purified material from cells harvested during exponential growth was immunoblotted as described. Molecular weight standards are indicated. Lanes 1 and 2, material from growth medium (1) and periplasmic (2) fractions from cells transformed with pSPTH37ZZ; lanes 3 and 4, material from growth medium (3) and periplasmic (4) fractions from cells transformed with pSPT1137ZZ.

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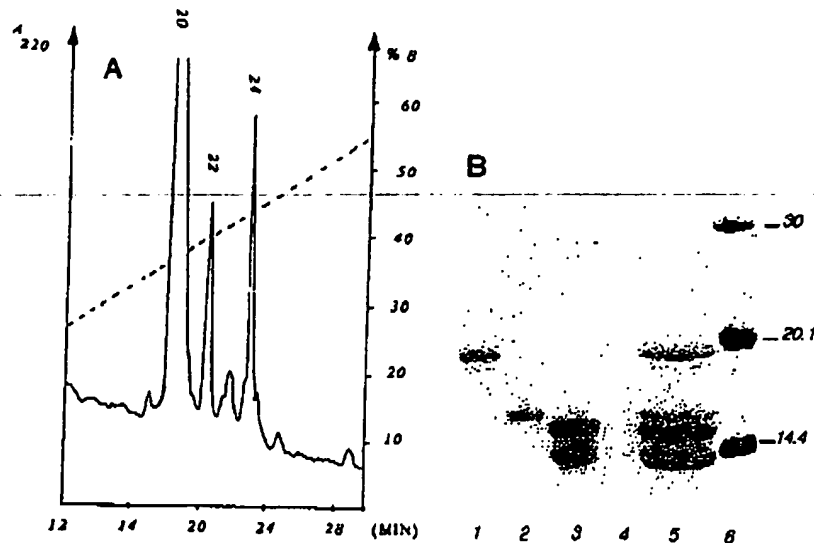


FIG. 6. Purification of expression products from the periplasmic fraction by reversed phase HPLC. IgG affinity-purified material was further purified by HPLC as described. (A) Chromatogram (A_{220}) of HPLC purification. Fractions used for SDS-PAGE are indicated by numbers. (B) The fractions indicated in A were subjected to SDS-PAGE and electroblotting, and the membranes were stained with Coomassie brilliant blue. Lane 1, fraction 24; lane 2, fraction 22; lane 3, fraction 20; lane 4, empty; lane 5, IgG affinity-purified sample of the periplasmic fraction before HPLC purification; lane 6, molecular weight standards.

terminal amino acid sequence analyses. The amino acid sequence obtained from the 19-kDa band was

Ser-Val-Ser-Glu-Ile-Gln-Leu-

This sequence corresponds to the amino acid sequence 11 to +7 of mature hPTH, identifying the 19-kDa band as correctly processed, probably full-length, hPTH(1-37)ZZ. The 15-kDa band amino acid sequence was

X-X-X-Leu-Gln-X-Val-His-Asn-Phe,

where X represents an unidentified amino acid residue. This sequence corresponds to amino acids +26 to +35 of mature hPTH, indicating a proteolytic cleavage between amino acids 25 and 26 in the hPTH part of the fusion protein. The 14-kDa band amino acid sequence was

X-X-X-Gln-His-Asp-Glu-Ala-

Val-Asp-Asn-Lys-Phe,

which corresponds to amino acids at position +40 to +53 that is in the ZZ part of the fusion protein.

Identical results were obtained when the corresponding protein bands were cut out from SDS-PAGE blots obtained directly after IgG affinity chromatography

(see e.g., Fig. 3, lane 4), demonstrating purification of these expression products in just two steps.

N-Terminal Amino Acid Sequence Analysis of Expression Products from Cells Containing pSPTH37ZZ

In order to analyze the major products of pSPTH37ZZ-containing bacteria, the 26-, 24-, and 22-kDa bands were cut from the filter and subjected to N-terminal amino acid sequence analyses. We were, however, not able to get an amino acid sequence for any of these bands. A probable reason for this is that these proteins are N-terminally blocked due to the presence of N-formylmethionine in an unprocessed signal sequence.

In conclusion, fair amounts of a correctly cleaved 19-kDa protein were present in the periplasmic fraction from pSPTH37ZZ-transformed cells harvested in exponential growth phase, although undesired proteolytic cleavage was observed at later stages of growth. The 19-kDa protein was also visible on immunoprobings of material from the growth medium (Fig. 4B, lane 4 and Fig. 5, lane 1), but the relative amount in the growth medium (as compared to the periplasmic fraction) was much lower than for some of the other ZZ-containing bands (e.g., the 14-kDa band in Fig. 4A, lanes 3 and 4). In comparison, cells transformed with plasmid pSPTH37ZZ generated mainly unprocessed hybrid

proteins that were localized the medium with very little present in periplasm.

DISCUSSION

The aim of this study was to seek a way to test signal sequences for secretion and correct processing of a foreign gene product in *E. coli*.

This report describes the successful expression of a correctly processed fusion protein containing the first 37 amino acids of hPTH fused to two IgG binding domains (ZZ) derived from *Staphylococcal* protein A. This ZZ part of the hybrid protein provided an efficient means for purification using IgG affinity chromatography. After just one additional purification step (SDS-PAGE) the products were pure enough to be characterized by amino acid sequencing.

The plasmid constructs used in this study should also be well suited to study the secretion and processing of proteins other than hPTH, if desirable employing other signal sequences than the one of protein A. In pKP43PTH (Fig. 1B) there are unique restriction sites both inside (*Ksa*I, *Kpn*I) and at the end of (*Ava*I) the signal sequence. In addition there is a useful *Asa*I site just in front of the signal sequence, making it easy to manipulate the signal sequence part of the construct. Unique restriction sites are also found at the front of the hPTH part (*Ava*I) as well as between the hPTH part and the ZZ part (*Xba*I, *Bam*HI). These sites could be used to insert genes other than hPTH, making it possible to study secretion and processing of other heterologous proteins in the same easy way as described here for hPTH.

Coomassie brilliant blue-stained SDS PAGE electrophoretograms of IgG affinity-purified material from exponentially growing cultures containing plasmid pSPTH37ZZ showed a different pattern of protein bands in the growth medium and periplasmic fractions. The periplasmic fraction contained a peptide with the theoretically correct molecular weight of hPTH(1-37)ZZ, around 19 kDa, although degraded forms migrating at 14, 15, and 15.5 kDa were also observed. The assumed correctly processed form was visible on immunoblots of both the periplasmic and the medium fractions. In general, cells harvested in exponential growth phase retained the expressed hybrid proteins in the periplasmic space and exported very little to the medium.

At the stationary phase of growth, however, the major part of the expression products had accumulated in the growth medium, although the 19-kDa band could not be detected in this fraction. Whether this is due to a selective degradation of the 19-kDa protein in the growth medium or whether the 19-kDa form is unable to pass the outer membrane is unclear at the moment. We attempted to sort this out by using two protease deficient *E. coli* strains, B121 and UT5600 (29). In these strains a

higher fraction of the expression products seemed to be unprocessed 22-kDa species, but the translocation and degradation patterns were largely unaltered (data not shown). Thus the use of protease mutants did not give any indications as to the fate of the 19-kDa molecule. Whether the use of different growth conditions or media may change the degradation or localization patterns remains to be seen.

N-terminal sequencing of the 19-kDa band revealed an amino terminus corresponding to the one in native hPTH. Sequencing of the 15-kDa band showed that the fusion protein was degraded before amino acid +26 in the hPTH region, while in the 14-kDa band cleavage seems to have occurred in the ZZ region, between Arg (+39) and Gly (+40). Interestingly, the main site of proteolytic degradation in the hPTH part is identical to that observed for hPTH(1-84) expressed as a secretory protein in *Saccharomyces cerevisiae* (28), but different from the main degradation site for hPTH(1-84) in *E. coli* (22). The known amino acid sequence of the fusion protein at positions 25, 26, and 27 is Arg-Lys-Lys-, thus the sequence of the 15-kDa band indicates proteolytic cleavage between Arg and Lys. This suggests that the enzyme responsible for the cleavage might be the OmpT protease, an outer membrane protein known to cleave between two basic amino acid residues (29). However, expression of pSPTI137ZZ in the OmpT negative strain UT5600 did not exclude this proteolytic degradation (data not shown).

In contrast, pSPTI137ZZ yielded almost exclusively putative unprocessed fusion proteins, both in the medium and in the periplasmic fraction. This indicates that the mutated signal sequence, as expected, is not cleaved by the signal peptidase. Thus, signal sequences in this expression system seem to be processed in the normal way by the *E. coli* cell.

From our results it is also apparent that the unprocessed fusion protein containing the mutated signal sequence is present in the growth medium, while the correctly processed form is localized mainly in the periplasm. The reason for this difference is at the moment unclear. One possible explanation is that the correctly processed form is degraded either in the medium or during passage through the outer membrane and that the presence of an uncleaved signal sequence might somehow protect against such degradation. In accordance with this notion our results indicate that the protein with the mutated signal sequence is less prone to degradation than the form with the wild type signal sequence. An alternative explanation is that proteins containing an uncleaved signal sequence might pass the outer membrane through a pathway that is not available to proteins without a signal sequence, resulting in a selective translocation of signal sequence containing forms to the growth medium.

hPTH(1-84) expressed in fusion with the same vari-

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ants of the protein A signal sequence is secreted and processed in the same way as our fusion protein, showing that the introduction of the protein A binding part does not influence the secretion and processing process [Ref. (22), and B. N. Kareem *et al.*, in preparation]. Altogether these findings indicate that the ZZ fusion-protein system is a useful model for a more general study of secretion and processing in *E. coli*.

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